Method for producing isotransgenic lines

The invention relates to a method for producing isotransgenic lines, characterized in that it comprises a step which makes it possible to target the genome which has received a T-DNA after transformation of a hybrid, and also to the commercial hybrids produced using these isotransgenic lines.

The expression "isotransgenic lines" is intended to mean isogenic transgenic lines, the isogeny being defined by the state of a genotype which differs from another only by a very small number of genes (1 or 2), often obtained by backcrossing. The isotransgenic lines according to the invention are characterized in that they have a fixed pure "line of interest" genotype over the entire genome and have stably integrated the T-DNA containing the transgene. They have the particularity of being free of any fragment which originates from the transformation line and which may constitute a genetic burden for the subsequent selection steps.

The term 'T-DNA', or transfer DNA, is intended to mean the DNA fragment containing the gene of interest and the sequences which allow its expression, which is transferred and integrated into the host genome during transformation.

In the context of the invention, two types of line will be distinguished: the transformation lines, or lines suited to transformation, of the A188 type for example; and the lines unsuited to transformation, hereinafter named lines of interest or agronomic lines. The term 'elite lines' will denote agronomic lines which have a considerable commercial potential at a given period. The elite lines have agronomic properties linked to the expression of phenotypic character traits relating in particular to their vegetative growth and to yield, these agronomic properties being technical characteristics which mark their commercial potential, i.e. their ability to be used in variety selection programs for placing commercial lines on the market.

The development of commercial hybrids generally involves several steps: (1) developing pure homozygous parental lines, using genetic material selected with respect to its potentialities; (2) crossing these lines so as to obtain hybrids and (3) evaluating the commercial potential of these hybrids, as a function of the phenotypic character traits acquired and of their hybrid vigor, or heterosis (vegetative growth and yield). This commercial potential is all the greater since the parental lines belong to varied heterotic groups and have advantageous characteristics hence the importance given to the research and development of improved parental lines, in particular by transgenesis.

However, the plant transformation techniques developed to date do not make it possible, today, to directly and efficiently transform the great majority of agronomic lines, including the elite lines, which are recalcitrant or unsuited to transformation (zero efficiency or efficiency of the order of 1/10 to 1/100), in particular in maize.

There have therefore been many studies relating, firstly, to the improvement of the *in vitro* culturing conditions for the transformation and regeneration steps and, secondly, to the search for a starting plant material which exhibits good transformation efficiency.

A greater knowledge of the environmental factors has thus made it possible to optimize the *in vitro* culturing conditions (transformation and regeneration) for adaptation to a greater number of genotypes; however, these improvements are not sufficient to overcome the recalcitrance of certain genotypes, in particular those of agronomic interest (Armstrong et al., 1992).

The choice of a plant material other than the pure lines, which are often recalcitrant to transformation, has therefore been proposed for developing methods, in particular in maize:

1) transformation of a donor line suited to transformation, of the A188 type (Armstrong et al., 1985), followed by successive backcrosses with pure

recipient lines unsuited to transformation, so as to obtain an 'isotransgenic' line (at least 5 to 6 backcrosses necessary if perfect isogeny desired). In practice, the lines resulting from these backcrosses are, at best, 'pseudoisogenic', since a fragment of the genome of the donor line is irreversibly linked to the transgene. Depending on its size and its nature, which depend on the processes of recombination, and/or on the limited availability of a molecular marker for sorting, said fragment may constitute a genetic burden which hinders the subsequent selection steps; in addition, the phenomena of recombination which would make it possible to reduce this burden are rare events, recombination between homeologous sequences being less efficient than between homologous sequences, rendering the great efforts of backcrossing obsolete. There is therefore a considerable risk of generating negative genetic effects in the final hybrid product, via the use of starting material of the A188 type suited to transformation.

2) direct transformation of a 'transformation line × line of agronomic interest' hybrid (Ishida et al., 1996). This hybrid, which combines the transformation/regeneration abilities and the agronomic characteristics of each of the parental lines, may appear to be a starting plant material more favorable to the ultimate production of commercial hybrids. However, the results obtained by Ishida et al. (1996)

show that the efficiency of transformation of the hybrid is clearly lower than that obtained for the line of the A188 genotype. Moreover, the risks of genetic burden in the final product are not avoided since the transgene may integrate on one or the other of the chromosomes of the hybrid (i.e. on the chromosome of the donor line of the A188 type in 50% of cases).

International application WO 98/32326 (Pioneer) proposes adjusting the two parameters - in vitro culturing conditions and plant material - so as, firstly, to improve the efficiency of transformation and, secondly, to make the basic method described by Ishida et al. (1996) applicable to lines other than A188. This team mentions an efficiency of transformation which is better than that obtained with the basic protocol, but this efficiency still remains low in the case of the lines unsuited to transformation.

There was, therefore, to date, no overall method or plant material for producing true 'isotransgenic' lines, integrating both the needs for high transformation frequency (which excludes the use of pure lines unsuited to transformation) and the necessity of having true isogeny for the transgenic lines produced (which, on the contrary, indicates the use of these pure lines in order to avoid any genetic burden originating from the transformation line).

The present invention makes it possible to provide an original solution to this problem by developing a novel method for producing isotransgenic lines which integrates a step which makes it possible to target the genome which has received the T-DNA. This method, based on the transformation of a hybrid, is, in fact, characterized by a step for selecting the primary transformants which have only integrated the T-DNA into the genome of the type unsuited to transformation (*a priori* 50% of the transformants). These selected transformants will lead to the creation of isotransgenic lines after backcrossing said transformants with the parental line of agronomic interest.

This step for selecting the transformants which have integrated the transgene into the genome of the type unsuited to transformation has never been suggested or described in the prior art. It is advantageous in that it makes it possible to ultimately produce a 'true' isotransgenic line, i.e. a line free of any fragment originating from the line suited to transformation, while at the same time keeping an acceptable level of efficiency of transformation. In addition, it makes it possible to improve the rapidity of transfer of the gene of interest into a pure genome, by decreasing the number of backcrosses required.

The method according to the invention, which integrates this step for selecting the primary

transformants, makes it possible to satisfy better the industrial requirements, in terms of rapidity and efficiency, which the methods described until now did not.

In addition, this method is of great value, in particular when the line of interest is part of many hybrid formulae or when it involves lines which dominate a considerable market. It allows the production of transgenic plants which may express, by way of examples, an antisense RNA, a ribozyme or a protein of interest conferring upon it resistance to diseases/pathogens and/or an improved agronomic or nutritional quality (amino acids, oil, starch, etc.).

The use of this method also makes it possible to vary the genetic sources of the lines from large heterotic groups, used as parental lines for the production of commercial hybrids. It also makes it possible to stack several transgenic characters in the agronomic lines without adding fragments which originate from the transformation line and which may be the subject of a genetic burden. This perspective is interesting in particular in terms of diversification of the genetic sources for the production of commercial hybrids which have conserved good or even improved hybrid vigor.

According to a first embodiment, the method for producing isotransgenic plant lines according to the invention comprises the steps of:

a) transforming the plant cells of a plant hybrid consisting of the crossing of two parental lines, a line of interest and a line suited to transformation, with a vector carrying a T-DNA containing a transgene;

b) selecting the hybrid primary transformants which have integrated said T-DNA only, into the genome of the line of interest;

c) backcrossing, with the parental line of interest, said primary transformants selected in b), and selecting the individuals derived from these backcrosses until isotransgenic lines are produced.

Among the hybrid primary transformants, those which exhibit a monolocus or monocopy insertion of the T-DNA are preferably pre-selected, i.e. those which have preferably integrated one copy of the transgene (monocopy) or possibly several copies in tandem, at the same chromosomal locus. The monocopy individuals are particularly preferred in that they are not affected by the phenomenon of gene extinction, known for multicopy insertions, and in that they allow simplified monitoring of the transgene. The expression 'insertion without extra-border sequence' is intended to mean transformants which have integrated only the T-DNA containing the transgene, without the transfer of

plasmid sequences outside the T-DNA, named extra-border sequences.

The monolocus, preferably monocopy transformants lacking any extra-border sequence may, in particular, be selected by the Southern technique with several restriction enzymes and several probes (Southern, 1975), making it possible to identify and characterize the insertion into the genome of the plant, and thus to differentiate the transformation events.

The method is characterized in that the step for selecting the hybrid primary transformants consists in identifying the genomic sequences adjacent to the T-DNA inserted, in order to determine the parent genome which has received said T-DNA.

For each primary transformant which has proved to be in accordance with the expected phenotype and which has been selected according to the criteria - monolocus or monocopy and absence of extraborders - the genomic sequences of the host adjacent to the T-DNA may be isolated and identified, for example via a method based on PCR (Polymerase Chain Reaction, Saiki Rk. et al., 1988), preferably IPCR (Inverse PCR, Does Mp. Et al., 1991); the aim being to identify the parental origin of the genome which has accepted the transgene (line of agronomic interest or transformation line).

Finally, the identification, for each transformant, of the genome of the parental line which

has integrated the T-DNA may, in particular, be based on demonstrating a polymorphism of the size of the restriction fragments (RFLP, Restriction Fragment Length Polymorphism, Burr B. et al., 1983) between the parental lines and the transformant, using, as probes, the adjacent genomic fragment(s) previously identified.

Alternatively, sequencing the genomic borders of the T-DNA and demonstrating SNP (Single Nucleotide Polymorphism) by comparison with the sequences of the parental lines may also make it possible to identify the recipient parent genome.

In addition, said identified adjacent genomic sequences may also be used as probes on a mapping population known to those skilled in the art, in order to identify the chromosome carrying the insertion and the position of this insertion, according to mapping techniques (for example Murigneux et al., 1993). This makes it possible to choose some markers around this position, to be used advantageously in the subsequent steps for selecting the backcrossed individuals.

The construction of expression vectors for the transformation (step a) is within the scope of those skilled in the art, following standard techniques, as described for example in Sambrook et al. (1989). Said expression vectors may contain a nucleotide sequence in the sense or antisense direction, encoding, for example, a protein of interest (agronomic, nutritional or therapeutic quality) or

protein for resistance to diseases and/or pathogens (herbicide, insecticide), a selection marker, an antisense RNA or a ribozyme, etc., and also regulatory sequences which allow its expression in the plant (promoter-constitutive, inducible or specific/addressing peptide/terminator). Weising et al. (1988) describe, in particular, promoters, polyadenylation sequences, selection marker genes, reporter genes, enhancers and introns which can be used in the context of the invention. Among the nucleotide sequences of interest, mention may be made of all the nucleic acids which make it possible to introduce or improve a beneficial character trait in the resulting transgenic plant. For example, the nucleic acid may encode proteins or antisense RNA transcripts so as to promote an increase in the nutritional values, in the yield, in the resistance to pathogens, to diseases, etc. Such genes are in particular described in patent applications WO 91/02071 and WO 95/06128.

By way of example, mention may be made of:

- the bacterial gene dapA for increasing the level of lysine;
- the gene for endotoxin Bt or for a protease inhibitor or for proteins extracted from bacteria such as Photorabus (WO 97/17432 & WO 98/08932), for resistance to insects;
- among the proteins or peptides of interest which confer novel properties of resistance to diseases,

mention will be made in particular of chitinases (WO 92/01792), glucanases (WO 93/02197), oxalate oxidase (WO 94/13790) or antibacterial and/or antifungal peptides, in particular the peptides of less than 100 amino acids rich in cysteins, such as plant thionins or defensins, and more particularly lytic peptides of any origins comprising one or more disulfide bridges between the cysteins and regions comprising basic amino acids, in particular the following lytic peptides: androctonin (WO 97/30082 and WO 99/09189), drosomicin (WO 99/02717), thanatin (WO 99/24594) or heliomycin (WO 99/53053). According to a particular embodiment of the invention, the protein or peptide of interest is chosen from fungal elicitor peptides, in particular elicitins (Kamoun et al., 1993; Panabières et al., 1995).

- the *bar* or *pat* gene which confers tolerance to bialaphos, a bacterial or plant gene encoding an EPSPS for resistance to the herbicide glyophosate (US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,312,910, US 5,633,435, US 5,627,061, US 5,310,667, WO 97/04103); the gene encoding glyophosate oxidoreductase (US 5,463,175), a bacterial or plant gene encoding a native, mutated or chimeric HPPD (WO 96/38567, WO 98/02562, WO 99/24585, WO 99/24586) which

confers tolerance to herbicides having HPPD as a target (diketones, isoxazoles, mesotrione, etc.);

- genes involved in the biosynthetic processes which lead to a change in the quality of the products of the transgenic plant, such as the genes encoding enzymes for the biosynthesis or degradation of starch (i.e. synthases, starch-branching enzymes, etc.); genes encoding grain storage proteins (i.e. subunits of glutenins, gliadins, hordeins); genes related to the strength of the grain in wheat (i.e. puroindolines).

- genes which modify the constitution of the modified plants, in particular the content and the quality of certain essential fatty acids (EP 666 918) or the content and the quality of the proteins, in particular in the leaves and/or the grains of said plants. Mention will be made, in particular, of the genes encoding proteins enriched in sulfur-containing amino acids (Korit, A.A. et al.; WO 98/20133; WO 97/41239; WO 95/31554; WO 94/20828; WO 92/14822). The function of these proteins enriched in sulfur-containing amino acids will also be to trap and store excess cystein and/or methionine, making it possible to avoid the possible problems of toxicity linked to an overproduction of these sulfur-containing amino acids by trapping them. Mention may also be made of genes encoding

peptides rich in sulfur-containing amino acids, and more particularly in cysteins, said peptides also having antibacterial and/or antifungal activity. Mention will be made more particularly of plant defensins, and also lytic peptides of any origin, and more particularly the following lytic peptides: androctonin (WO 97/30082 and WO 99/09189), drosomicin (WO 99/02717), thanatin (WO 99/24594) or heliomycin (WO 99/53053).

- genes for artificial male sterility (i.e. barnase, and PR-glucanase under the control of a suitable promoter) may also be used for the production of hybrid seeds.

The nucleic acid sequences of interest may also be introduced as a genetic tool to generate mutants and/or to assist the identification, molecular labeling or isolation of plant gene segments. Other examples are described in Weising et al.

The expression vector comprising the nucleic acid sequence of interest to be introduced into the plant will generally comprise a selection marker or a reporter gene, or both, in order to facilitate the identification or selection of the transformed cells. Alternatively, the selection marker may be carried by a second vector and used in cotransformation. These sequences must be flanked by suitable regulatory sequences to allow their expression in the plants. The selection markers are well known to those skilled in

the art and include, for example, genes for resistance to antibiotics and to herbicides. Particular examples are described in Weising et al. or patent applications EP 242 236, EP 242 246, GB 2 197 653, WO 91/02071, WO 95/06128, WO 96/38567 or WO 97/04103. A preferred selection marker is hygromycin B phosphotransferase (hpt), which may be derived from E.coli. Mention may also be made of the gene for aminoglycoside phosphotransferase of transposon n5 (AphII) which encodes resistance to the antibiotics kanamycin, neomycin and G418, and also the genes which encode resistance or tolerance to glyophosate, bialaphos, methotrexate, imidazolinones, sulfonylureas, bromoxynil, dalapon and derivatives. The selection marker genes which confer tolerance to herbicides are also of commercial use in the resulting transformed plants. The reporter gene is generally a gene which is not present or expressed in the recipient organism or tissue and which encodes a protein, the expression of which is revealed by detectable properties, such as a phenotypic change or an enzymatic activity. Examples are given in Weising et al. Among the preferred genes, mention may be made of the chloramphenicolacetyl-transferase (cat) gene from tn9 of *E.Coli*, the beta-glucuronidase (gus) gene at the uidA locus of *E.Coli*, the green fluorescent protein (GFP) gene from *Aequoria victoria*, and the luciferase gene from *Photinus pyralis*.

The regulatory sequences also include constitutive promoters, inducible promoters, tissue- or organ-specific promoters, or developmental stage-specific promoters, which can be expressed in plant cells. Such promoters are described in Weising et al.

Mention may also be made of:

- the regulatory sequences for the T-DNA of *A. tumefaciens*, including mannopine synthase, nopaline synthase and octopine synthase;
- the maize alcohol dehydrogenase promoter;
- light-induced promoters, such as the gene for the ribulose biphosphate carboxylase small subunit from a variety of species and the promoter of the gene for the chlorophyll a/b binding protein;
- the histone promoters (EP 507 698), optionally combined with the first intron of rice actin (WO 99/34005);
- the maize ubiquitin 1 promoter (Christensen et al., 1996);
- the 35S promoter of the cauliflower mosaic virus, or the 19S promoter or advantageously the constitutive double 35S promoter (pd35S), described in the article by Kay et al., 1987;

- the pCRU promoter of the radish cruciferin gene, which allows expression of the associated sequences only in the seeds (or grains) of the transgenic plant obtained (Depigny-This et al., 1992);
- the pGEA1 and pGEA6 promoters corresponding to the 5' noncoding region of the GEA1 and GEA6 seed storage protein genes, respectively, from arabidopsis thaliana (Gaubier et al., 1993), which allow specific expression in the seeds;
- the rice actin promoter followed by the rice actin intron (pRA-RAI) contained in the pAct1-F4 plasmid described by McElroy et al., 1990;
- the wheat HMWG (high molecular weight glutenin) promoter by Robert et al., 1989;
- the promoters regulated during development, such as the maize waxy, zein or bronze promoters;
- the organ-specific promoters or developmental stage-specific promoters, such as the alpha-tubulin promoter described in US 5,635,618;
- the promoter of the maize zein gene (Pzéine) which allows expression in the albumen of maize seeds (Reina et al., 1990);

- the N promoter of a maize genomic clone, the cDNA of which is referenced in the publication by Shen et al. (1994).

Use may also be made of a regulatory promoter sequence specific for particular regions or tissues of plants, and more particularly seed-specific promoters (Datla, R. et al., 1997), especially the promoters for napin (EP 255 378), for phaseolin, for glutenin, for heliantinin (WO 92/17580), for albumin (WO 98/45460), for oelosin (WO 98/45461), for ATS1 or for ATS3 (WO 99/20775).

Use may also be made of an inducible promoter advantageously chosen from the promoters for phenylalanine ammonia lyase (PAL), for HMG-CoA reductase (HMG), for chitinases, for glucanases, for proteinase inhibitors (PI), for PR1 family genes, for nopaline synthase (nos) or for the vspB gene (US 5 670 349), the HMG2 promoter (US 5 670 349), the apple beta-galactosidase (ABG1) promoter or the apple aminocyclopropane carboxylate synthase (ACC synthase) promoter (WO 98/45445).

Other elements, such as introns, enhancers, polyadenylation sequences and derivatives, may also be present in the nucleic acid sequence of interest, in order to obtain to improve the expression or functioning of the transforming gene. By way of example of an enhancer, there is the translation activator of the tobacco mosaic virus (TMV) described in application

WO 87/07644, or of the tobacco etch virus (TEV) described by Carrington & Freed (1990). Among the introns which can be used, the Adh1S first intron from maize may be placed between the promoter and the coding sequence of a nucleic acid sequence of interest. This intron, included in a genetic construct, is known to increase the expression of a protein in maize cells (Callis et al., 1987). Use may also be made of the first intron of the maize shrunken-1 gene (Maas et al., 1991), the first intron of the castor pea catalase gene (cat-1) (Ohta et al., 1990); the second intron of the potato catalase ST-LS1 gene (Vancanneyt et al., 1990); the intron of the tobacco yellow dwarf virus (DSV) (Morris et al. 1992); the rice actin-1 (act-1) intron (McElroy et al. 1990) and intron 1 of triose phosphate isomerase (TPI) (Snowden et al., 1996). However, sufficient expression may often be obtained without an intron (Battraw et al., 1990).

The expression vector may also comprise sequences encoding a transit peptide, so as to lead the protein encoded by the heterologous gene into the chloroplasts of the plant cells. These transit peptides, which are well known to those skilled in the art, may include simple transit peptides or multiple transit peptides obtained by combining sequences encoding at least two transit peptides. The transit peptide may be simple, such as an EPSPS transit peptide (described in US patent 5,188,642) or a transit peptide

of the ribulose-biscarboxylase/oxygenase small subunit (RuBisCO ssu) from a plant, optionally comprising some amino acids from the N-terminal portion of the mature RuBisCO ssu (EP 189 707) or a multiple transit peptide comprising a first plant transit peptide fused to a portion of the N-terminal sequence of a mature protein located in the plastid, fused to a second plant transit peptide as described in patent EP 508 909, and more particularly the optimized transit peptide comprising a transit peptide of sunflower RuBisCO ssu fused to 22 amino acids of the N-terminal end of maize RuBisCO ssu fused to the transit peptide of maize RuBisCO ssu as described with its coding sequence in the patent EP 508 909. A preferred transit peptide is Optimized Transit Peptide (OTP) described in the patent US 5,635,618.

Plant cells from the hybrid may be transformed using techniques known to those skilled in the art.

Mention may in particular be made of direct gene transfer methods, such as direct microinjection into plant embryoids (Neuhaus et al., 1987), infiltration under vacuum (Bechtold et al., 1993) or electroporation (Chupeau et al., 1989), or alternatively direct precipitation using PEG (Schocher et al., 1986) or bombardment with a particle gun (Fromm M. et al., 1990).

It is also possible to infect the plant with a bacterial strain, in particular of *Agrobacterium*. According to one embodiment of the method of the invention, the plant cells are transformed with a vector according to the invention, said cellular host being capable of infecting said plant cells, allowing the integration, into the genome of the latter, of the DNA sequences of interest initially contained in the genome of the abovementioned vector. Advantageously, the abovementioned cellular host used is *Agrobacterium tumefaciens*, in particular according to the method described in the article by An et al. (1986), or alternatively *Agrobacterium* rhizogenes, in particular according to the method described in the article by Jouanin et al., 1987.

Preferentially, the plant cells are transformed by transferring the T region of the tumor-inducing extrachromosomal circular plasmid Ti of *Agrobacterium tumefaciens*, using a binary system (Watson et al.). To do this, two vectors are constructed. In one of these two vectors, the T-DNA region has been removed by deletion, with the exception of the right and left borders, a marker gene being inserted between them to allow selection in the plant cells. The other partner of the binary system is a helper Ti plasmid, which is a modified plasmid which no longer has any T-DNA but still contains the vir

virulence genes required for transforming the plant cell. This plasmid is maintained in *Agrobacterium*.

Preferentially, the plant cells are transformed with *Agrobacterium tumefaciens* according to the protocol described by Ishida et al (1996), in particular using immature embryos which are 10 days post-fertilization.

Alternatively, it is possible to use the method for transforming immature embryos described in international application WO 98/32326, or the method for transforming inflorescences of monocotyledon plants, described in patent application WO 99/67357.

The two parental lines of the hybrid are thus chosen: for one, with respect to its ability to be transformed (parental transformation line) and for the other, with respect to its polyvalence or its commercial importance with regard to the market (parental line of interest).

Currently, the line which has the greatest ability to be transformed with *Agrobacterium* is the A188 line; it is this line which is generally used for producing a transformant. Among the known commercial elite lines and transformation lines, mention may be made in particular of those described by Ishida et al (1996) and Pioneer (WO 98/32326).

Among the cells which can be transformed according to the method of the invention, mention may be made, by way of examples, of the cells of large crop

plants (maize, wheat, rapeseed, sunflower, pea, soybean, barley, etc.) or of vegetable plants and flowers.

The steps for transformation (a) and selection (step b- method according to the invention) described above have made it possible to select transformants which have integrated the transgene into the genome of the type not suited to transformation. Said selected transformants contain 50% of the genome of the parental transformation line and 50% of the genome of the parental agronomic line.

The reconversion to a line with a fixed pure genome of interest (step c), involves successive backcrosses with the parental line of interest and selection of the individuals obtained according to the conventional method of phenotypic analysis or, preferentially, selection assisted by markers (Hospital et al., 1992).

This selection is based in particular on the following criteria:

(i) variability around the site of integration of the transgene, with elimination of any linked fragment originating from the donor transformation line (selection of recombination events). The desired genetic recombination is selected on one side of the gene at one generation of backcross and on the other side at the following generation.

(ii) search for the best genome of interest ratio (ratio of the % genome of agronomic interest to the % overall genome) for the entire genome.

Step (i) proves to be limiting when the transgene is inserted into a genome of the A188 type for example, since it is necessary to remove any fragment originating from this transformation line by selecting the recombination events closest to the transgene (rare events). This requires: performing the backcrossing steps on a large number of plants in order to select at least one plant correctly recombined on both sides of the insertion (2nd backcross); waiting for an additional backcross in order to apply, on a sufficient number of plants, a selection pressure over the entire genome. While it is possible to ultimately produce plants with a genome which is fixed at more than 99% genome of interest from the 4th backcross, these plants will remain, at best, pseudoisogenic at the site of insertion of the transgene.

When the transgene is inserted into a genome of the agronomic type (parental line of interest), and the primary transformants are selected for this characteristic according to the invention, step (i) of selection of rare recombination events is no longer necessary. Consequently, a potential decrease is obtained in the number of backcrosses necessary and/or in the number of individuals to be tested and/or in the number of markers for the selection, as will be

described in Example 4. The lightening of the overall method for reconversion to the pure genome of interest adds to the major benefit of the invention, which is the production of true isogeny for the transgenic lines produced.

A subject of the invention is also a method in which the individuals in which the chromosome which has received the T-DNA has conserved a genotype entirely of the line of interest type, and which have a genome of interest to entire genome ratio of at least 75%, are selected from the first backcross in c).

The use of the method to introgress several transgenic characteristics into a plant, without adding fragments linked to the transgene which may be the subject of a genetic burden, also falls within the context of the invention.

The invention also relates to a method which makes it possible to target the parent genome which has received a T-DNA after transformation of a hybrid, comprising the identification of the genomic sequences adjacent to the T-DNA inserted.

A subject of the invention is also any transgenic plant or part of a plant, in particular seed, obtained according to the invention in one or other of the steps described above.

The true isotransgenic lines produced from hybrid transformants, characterized in that they have a fixed pure 'line of interest' genotype over the entire

genome and have stably integrated the T-DNA containing the transgene, are also part of the invention. In particular, the true isotransgenic lines produced according to the invention are elite lines.

According to another embodiment, the method according to the invention is characterized in that it comprises a subsequent step of crossing between the isotransgenic line according to the invention and another line of interest, in particular another isotransgenic line according to the invention containing a different transgene, for producing commercial hybrids.

The invention also relates to the commercial hybrids thus produced.

The figures and examples below illustrate the invention without limiting the scope thereof.

LEGENDS TO THE FIGURES

Figure 1: plasmid map of a construct derived from pBIOS273

Figure 2: demonstration, by RFLP analysis on the primary transformants, of the parental genome which has received the T-DNA.

EXAMPLES

The transformation of maize, by way of example, may in particular be carried out according to the protocol of Ishida et al (1996) which uses the natural properties of *Agrobacterium tumefaciens* and the strategy of the binary system (Hiei et al., 1994).

Example 1: Preparation of vectors

The superbinary plasmid is the result of homologous recombination between an intermediate vector carrying the T-DNA containing the gene of interest and/or the selection marker, and the Japan tobacco pSB1 vector (EP 672 752) which contains: the virB and virG genes of the pTiBo542 plasmid present in the supervirulent strain A281 of *Agrobacterium tumefaciens* (ATCC 37349) and a homologous region found in the intermediate vector, which allows this homologous recombination.

The intermediate vector for introducing the gene of interest is the pBIOS 273 vector. This vector was generated in 2 main steps:

- cloning the BspDI/XhoI fragment (pAct-Bar-terNos) of the pDM 302 vector (Cao et al., 1992) into the pSB12 vector (Komari T. et al., 1996) digested with SmaI/BspD1: the pDM302 vector is digested with the XhoI enzyme (single site on the vector), thus generating 5' protruding sticky ends. These ends are made blunt after treatment with Klenow. A second digestion is then carried out with BsPD1 (sticky ends). The joining of the 'blunt' XhoI and SmaI sites makes it possible to recreate the XhoI cleavage site (at position 2363). These various steps allow oriented cloning into pSB12 and the resulting vector is named pBIOS 272.
- deleting the XhoI site at position 3363 of the pBIOS 272 vector by partial digestion with XhoI and the

action of the DNA Polymerase I large fragment. The vector obtained, which has a single XhoI site, is named pBIOS 273.

A large number of sequences encoding a gene of interest may be cloned into this pBIOS 273 vector, for the purposes of the invention, according to cloning techniques well known to those skilled in the art (Figure 1).

The intermediate vector is introduced into the cells of *A. tumefaciens* strain LBA 4404 (Hoekema et al. 1983) containing the pSB1 vector by electroporation according to well-known methods. The agrobacteria containing the superbinary vectors are selected on YT $CaCl_2$ medium in the presence of antibiotics (the genes for resistance to which are carried, respectively, by the plasmids of the various types), for example tetracyclin and spectinomycin at a concentration of 50 mg/l. Only the recombinant superbinary plasmids will carry resistance to spectinomycin (gene initially on the intermediate plasmids, which do not have an origin of replication in *Agrobacterium*, and which are, consequently, incapable of replicating in this bacterium). These plasmids are then characterized by enzymatic restriction and Southern analysis.

Example 2: Maize hybrid transformation

a) Production of the hybrid

The lines chosen to produce the hybrid to be transformed (line A188 and line of interest) are sown

under glass and then cultivated in a phytotron or under glass after repotting. The plants are cultivated in peat and watered daily with a nutrient SuperPlantora solution (NPK content: 14-10-14 + 3% of MgO). They are subjected to a photoperiod of 16::8 and to a light intensity of 3 to 4000 Lux. The mean temperature is 25°C. As soon as the ear emerges, it is covered with a paper bag in order to avoid any contamination with foreign pollen. This bag is kept in place until the ear is harvested.

The hybrid embryo is produced either by pollinating the A188 line with pollen from the elite line, or by pollinating the elite line with pollen from A188.

9 to 10 days after pollination, the ear is observed in order to determine the size of the embryos. If the size is between 1 and 1.2 mm, the ear is harvested and the embryos are immediately removed to be transformed according to the protocol described by Ishida et al. (1996).

b) Transformation and regeneration

The transformation protocol described by Ishida et al. (1996) was chosen in the context of this example; all the media used are referenced in that protocol. The transformation begins with a coculturing phase in which the immature embryos of the maize plants are brought into contact, for at least 5 minutes, with *Agrobacterium tumefaciens* LBA 4404 containing the

superbinary vectors. The embryos are then placed on LSAs medium for 3 days in the dark at 25°C. A first selection is performed on the transformed calluses: the 'embryo-calluses' are transferred onto LSD5 medium containing phosphinotricine at 5 mg/l and cefotaxime at 250 mg/l (elimination or limitation of contamination with *Agrobacterium tumefaciens*). This step is carried out for 2 weeks in the dark at 25°C. The second selection step is carried out by transferring the embryos which have developed on LSD5 medium, onto LSD10 medium (phosphinotricine at 10 mg/l) in the presence of cefotaxime, for 3 weeks under the same conditions as previously. The third selection step consists in excising the type I calluses (fragments of 1 to 2 mm) and in transferring them into the dark for 3 weeks at 25°C on LSD 10 medium in the presence of cefotaxime.

The regeneration of the plantlets is carried out by excising the type I calluses which have proliferated and transferring them onto LSZ medium in the presence of phosphinotricine at 5 mg/l and cefotaxime, for 2 weeks at 22°C and under continuous light.

The plantlets which have regenerated are transferred onto RM + G2 medium containing 100 mg/l of augmentin for 2 weeks at 22°C and under continuous illumination for the development step. The plants obtained are then transferred to the phytotron for the purpose of acclimatizing them.

Alternatively, the protocol described in patent application WO 98/32326 may be used to transform immature maize embryos.

Example 3: Selection of transformants which have integrated the transgene onto the genome of interest (parental genome not suited to transformation)

a) Selection of monocopy transformants lacking undesirable plasmid sequence

Among the primary transformants, those which exhibit a monolocus or monocopy insertion without undesirable plasmid sequence are therefore preferentially chosen. The Southern technique with several restriction enzymes and several suitable probes (Southern, 1975) may in particular be used to identify and characterize the insertion into the genome of the plant, thus making it possible to differentiate the transformation events. This methodology in fact makes it possible to demonstrate individual differences in the size of the restriction fragments obtained with a given enzyme and a given probe, corresponding to defined positions on the genome.

By way of example, the protocol described in Sambrook et al. (1989) may be used. The genomic DNA is extracted from leaves of the primary transformants according to a CTAB extraction protocol (Dean C. et al., 1992). This DNA is then digested according to well-known molecular biological techniques, with a

restriction enzyme which cleaves at least once within the T-DNA. Using suitable probes which hybridize on either side of the cleavage site, it is thus possible to characterize the insertion on both internal sides - right and left borders - of the T-DNA according to the suitable procedure. For the same probe and for several individuals, differences observed in the size of the bands reflect different insertion sites. The DNA fragments obtained are separated on a 0.9 to 1% agarose gel and then transferred onto a Hybond N+ membrane (Amersham). The DNA of the intermediate plasmid comprising the T-DNA carrying the gene of interest and the selection marker is included in the analysis as a control. The membrane is hybridized with probes homologous to the sequences of the transgene studied:

- a probe specific for the selective marker gene, named S1;
- a probe specific for the gene of interest, named S2;
- two probes, named ex RB and ex LB (for extra Right Border and extra Left Border), which are used jointly for the hybridization. This hybridization makes it possible to eliminate the plants containing sequences external to the T-DNA (extra-border sequences). A correct integration supposes that only the T-DNA is inserted into the host genome, without extra-border sequence. Since the sequences of the basic plasmids are known (pBIOS273 derived from pSB12), the ex RB and ex LB probes are obtained by amplification with

oligonucleotides specific for the extra-border plasmid regions of the T-DNA, RB2 and 3 for ex RB, and LB2 and 3 for ex LB.

SEQ ID No. 1 Oligo RB2: 5' ATCATCCTGTGACGGAACTTTG 3'

SEQ ID No. 2 Oligo RB3: 5' AAGGGCGTGAAAAGGTTTATCC 3'

SEQ ID No. 3 Oligo LB2: 5' GCTCGGCACAAAATCACCAC 3'

SEQ ID No. 4 Oligo LB3: 5' CATAGTTCTCAAGATCGACAGC 3'

The plants selected at the end of this molecular analysis show no signal for hybridization with the ex RB and ex LB probes and, if possible, exhibit a single band with each of the S1 and S2 probes, reflecting a simple monocopy insertion (one copy of the selection gene and one copy of the gene of interest). According to the procedure selected, differences in the size of the bands between plants will reflect different insertions corresponding to different transformation events.

b) Identification of the genomic sequences adjacent to the inserted T-DNA

For each primary transformant which has proved to be in accordance with the expected phenotype and which has been selected according to the criteria - monolocus or monocopy and absence of extra-border sequences - the genomic sequences adjacent to the T-DNA can be isolated and identified, for example via a method based on PCR, the aim being to identify the parental origin of the genome which has received the transgene (line of interest or transformation line).

Several techniques based on PCR may be used, for example PCR walking (Devic et al., 1997); preferably, the commercial Universal GenomeWalker kit from the company Clontech may be used in the context of this invention. The following protocol may be followed, based on the instructions for using this kit: the DNA of the primary transformants previously selected is digested separately with 5 restriction enzymes (enzymes which generate DNA fragments having blunt ends); the enzymes used may be those recommended by the manufacturer, with a 6-base pair, bp, restriction site - DraI, Eco RV, PvuII, ScaI and StuI, or other enzymes specific for restriction sites with 4 or 5 bp. For each sample, the fragments generated are then ligated at both ends to the GenomeWalker adaptor provided with the kit. Each sample is then separated in two (samp1 and samp2) in order to be able to determine the contiguous genomic sequences at the two borders of the T-DNA. The recovery of the genomic regions flanking the two borders of the T-DNA makes it possible not only to confirm the result of the integration but also to facilitate the identification of the recipient parent and to enable verification of the genetic mapping if necessary.

Two types of oligonucleotide are designated for carrying out the successive PCR amplifications: the AP, for adaptor primer, oligos provided by the kit; and the GSP, for gene-specific primer, oligos, the choice

of which depends on the sequence of the vector derived from pSB12 and on the parameters defined in the instructions for using the kit. Among the GSP oligonucleotides which may be used according to the invention, mention may be made of the oligonucleotides identified by the MacVector program (version 6) using the characteristics described in the table below.

Name	Sequence	Size	Tm (°C)	Position (pBIOS273)	% GC
GSPLB1	ID NO 5	29	71.3	3020	58.6
GSPLB2	ID NO 6	29	64.9	3081	41.4
GSPLB3	ID NO 7	28	70.1	3021	57.1
GSPLB4	ID NO 8	27	70.1	3018	59.3
GSPLB5	ID NO 9	27	70.1	3019	59.3
GSPLB6	ID NO 10	27	68.7	3022	55.6
GSPLB7	ID NO 11	27	63.3	3064	40.7
GSPLB8	ID NO 12	27	63.3	3077	40.7
GSPLB9	ID NO 13	26	68.7	3019	57.7
GSPLB11	ID NO 14	26	68.7	3023	57.7
GSPLB13	ID NO 15	26	63.0	3078	42.3
GSPRB1	ID NO 16	29	67.5	571	48.3
GSPRB2	ID NO 17	29	67.5	592	48.3
GSPRB3	ID NO 18	29	67.5	655	48.3
GSPRB4	ID NO 19	29	66.2	656	44.8
GSPRB5	ID NO 20	28	67.4	570	50
GSPRB6	ID NO 21	28	66.1	591	46.4

Name	Sequence	Size	Tm (°C)	Position (pBIOS273)	% GC
GSPRB7	ID NO 22	28	66.1	654	46.4
GSPRB8	ID NO 23	28	66.1	655	46.4
GSPRB9	ID NO 24	27	67.4	569	51.9
GSPRB10	ID NO 25	27	66.0	590	48.1
GSPRB11	ID NO 26	27	70.1	614	59.3
GSPRB12	ID NO 27	27	64.6	653	44.4
GSPRB13	ID NO 28	27	64.6	654	44.4
GSPRB14	ID NO 29	26	65.9	568	50
GSPRB15	ID NO 30	26	64.5	589	46.2

A first PCR amplification, using, for example, the oligos of the GSPLB or GSPRB type depending on whether they are specific for an internal sequence of the T-DNA on the RB or LB side, is carried out as follows:

- with the AP1 oligo specific for the adaptor and the GSPRBx oligo for sample 1,
- with the AP1 oligo and the GSPLBx oligo for sample 2.

The amplification products are diluted and then subjected to a second amplification:

- with the AP2 oligo specific for the adaptor and the GSPRBy oligo for sample 1,
- with the AP2 oligo and the GSPLBy oligo for sample 2.

These GSP oligonucleotides can be used in particular for all the vectors derived from pBIOS273, in which

only the sequence of the gene of interest will be replaced.

Sequences specific for the genes inserted into the T-DNA may also be used.

The PCR amplification products obtained are analyzed on the gel and those which are greater than 200-300 bp are preferentially chosen. These PCR amplification products, which contain a known sequence component (between oligo GSP and border of the T-DNA) and an unknown genomic sequence component (between oligo AP and border of the T-DNA), are then cloned, for example into the pGEM-T plasmid vector (Promega) according to the supplier's recommendations, and then sequenced with the universal direct and reverse oligonucleotides. Where appropriate, internal oligonucleotides may be used to complete the data. It is thus possible to generate probes 'specific' for the host genomic sequence bordering the T-DNA.

c) Identification of the recipient parent genome

This last step, which leads to the identification, for each transformant, of the genome of the parental line which has integrated the T-DNA, may in particular be carried out according to the following protocol (Sambrook et al. 1989). The recovered borders are used as probes and hybridized on an electrophoresis gel transfer containing the DNA, digested separately with various restriction enzymes, of the various

transformants and of the two parental lines. The demonstration of a restriction fragment length polymorphism (RFLP) for fragments homologous to the probe, and therefore to the insertion locus, between the parental lines, makes it possible to define the recipient parent by comparison with the profile of the transformant, which is heterozygous for the insertion. The expression 'heterozygous (hemizygous) for the insertion' is intended to mean that the hybrid transformant carries the T-DNA containing the transgene on only one of the two chromosomes. Integration into the parental line of interest therefore results in an RFLP with respect to the line of interest but not with respect to the other parent. In the case of a simple insertion into the line of interest, the profile of the primary transformant is characterized by two bands: a band identical in size to that of the transformation parent and a band different in size from that of the parent of interest. By way of example, Figure 2 gives the expected profiles for the parents and the transformant, in the case of an insertion into the chromosome of one or the other of the parents (2 cases).

The identification of the recipient parent may also be obtained according to another alternative, which consists in using the sequencing data from the genomic borders of the T-DNA obtained in b) to demonstrate SNPs (single nucleotide polymorphisms)

between the parental lines. After cloning the recovered adjacent genomic sequence into pGEM-T and determining the complete sequence, oligonucleotides can be designated on the sequence and used for new PCR amplifications on the parental lines and the transformant. If a nucleotide polymorphism exists between the two parents for the genomic portion which has been used to designate the oligonucleotides, an insertion into the line of interest will result in amplification for the line of interest and the transformant and no amplification for the other parent. If, on the other hand, no polymorphism is detected (amplification of the same fragment in the two parental lines, in the case of a conserved genomic fragment), it will be necessary to sequence said fragments amplified in the parental lines. The comparative analysis of the adjacent genomic sequences of the transformant, identified and sequenced in b), will then determine the parent which has received the transgene. According to one method or the other, it will be possible to differentiate the pseudoisogenic lines obtained using the methods described in the prior art from the true isotransgenic lines obtained according to the invention.

Example 4: Backcrosses with the parent of interest and selection of individuals until isotransgenic lines are produced

The previous steps made it possible to select transformants which had integrated the transgene into the genome of interest; moreover, said transformants contain 50% of parent transformation genome and 50% of genome of interest.

Preferentially, the selection of the plants derived from the backcrosses with the parental line of interest is assisted by markers according to known methods, in particular that described by Ragot et al. (1995).

As a comparison and demonstration of the advantages of selecting primary transformants according to the invention, the case of a process of selection after insertion of the transgene into the genome of the A188 type will be described in preamble.

<u>The case of an insertion on a chromosome of the A188 type (no selection of primary transformants)</u>

Conventionally, the desired genetic recombination is selected on one side of the gene at one generation of backcross and on the other side at the following generation. Since the size of the maize genome is estimated at 2000 centimorgans (cM), the recombination events to be selected during the first two backcrosses (BC1 and BC2), in order to transfer only 1/1000th of the non-elite genome linked to the transgene, will have to be located at 1 cM either side of the insertion, respectively.

The selection of the recombination events, assisted by predefined markers (close to the transgene), is carried out in BC1 and BC2 on a number of plants calculated as follows: the number N of plants to be tested in order to obtain 1 plant recombined at 1 cM with a probability of 95%, is N = (log0.05)/(log(1-0.01)) ~ 300 plants, according to the well-known law of probability. Knowing that a single recombined plant is obtained at the end of these two backcrosses, it appears, moreover, to be difficult to apply a selection for an optimal genome of interest ratio. The genome of interest ratios expected in BC1 and BC2 are, on average, 75% and 87.5%, respectively. A selection pressure over the entire genome may really be applied only in BC3, with about a hundred markers distributed over the entire maize genome (University of Missouri databank).

The reconversion to genome of interest, for this particular case, requires carrying out the backcross steps on a large number of plants (selection of rare events on the carrier chromosome) and waiting for at least the 3rd backcross to exert the second selection pressure, over the entire genome. Finally, the plants ultimately produced remain, at best, pseudoisogenic (potential genetic burden).

Backcross	Selection of recombination event at the site of insertion of the transgene		Characterization of the entire genome for the genotype of interest	
	No of plants tested	Plant with correct recombination	No of markers to be tested on residual heterozygous genome	% genome of interest
BC1	300	1	100	75
BC2	300	1	50	87.5
BC3	100		25	96.8
BC4	100		7	99.2

Total number of tests:

(300x1)+100+(300x1)+50+(100x25)+(100x7) = **3950 tests,** to select **1 plant** which is **pseudoisogenic** at the site of the transgene (carries 1/1000 of the A188 genome linked to the transgene, i.e. on average 50 to 80 genes, the maize genome having, on average, 50 000 to 80 000 genes), and with a genome **fixed at more than 99% genome of interest in BC4.**

The case of an insertion on a chromosome of the parental line of interest (selection according to the invention of the corresponding primary transformant, with backcrosses with the parent of interest)

Depending on the orientations chosen as a function of the priorities given to the biotechnology and/or the production/yield and/or the selection,

several selection schemes are possible, integrating, where appropriate, a preselection on the chromosome which has received the insertion. Unlike the case of an insertion into a genome of the A188 type, recombination events located far from the transgene or no recombination on the chromosome which has received the transgene will be selected here.

By way of examples, the following options may be mentioned.

Option 1: No selection at the site of integration of the transgene; selection gene of interest ratio from BC1.

Backcross	Selection of recombination event at the site of insertion of the transgene		Characterization of the entire genome for the genotype of interest	
	No of plants tested	Plant with correct recombination	No of markers	% genome of interest
BC1	100		100	87.5
BC2	100		25	≈ 100
BC3	100		7	≈ 100

Total number of tests: (100x100)+(100x25)+(100x7) = **13200 tests**, to select **1 plant truly isogenic** at the site of the transgene and with a **genome fixed at ≈ 100% genome of interest in BC3** (gain of one backcross).

Option 2: Selection in BC1 of the individual having the chromosome carrying the transgene which is entirely of the line of interest type; selection in BC3 for the gene of interest ratio.

Since the chromosome on which the T-DNA is inserted is known (mapping), it is possible to select, in BC1, using 10 markers distributed over this chromosome, a plant for which the integrity of the recipient chromosome of interest is conserved (no recombination event). Since it is known that the chromosomes are, on average, 200 cM in length, the probability of having a plant with no recombination event is $P=(0.99)^{200}\sim0.14$. The number N of plants to be tested with a 95% probability of obtaining it is $N = (\log 0.05)/(\log(1-0.14))\sim20$.

The plant thus selected will be backcrossed with the parent of interest until total reconversion (100%) to the genome of interest is obtained over the entire genome.

Backcross	Selection of elite genome integrity on the chromosome carrying the transgene (10 markers)		Characterization of the entire genome for the genotype of interest	
	No of plants tested	Plant with elite chromosome	No of markers	% genome of interest
BC1	20	1		75
BC2	100			87.5
BC3	100		25	≈ 100
BC4	100		7	≈ 100

Total number of tests: (20x10)+(100x25)+(100x7) = **3400 tests**, to select **1 plant truly isogenic** at the site of the transgene and with a **genome which is fixed at ≈ 100% genome of interest from BC3**.

Depending on the schemes, a decrease in the number of backcrosses (rapidity of production), or a decrease in the number of plants to be used for the backcrosses, will be sought in addition to the isogeny actually obtained according to the method of the invention.

Example 5: Production of commercial hybrids

It is possible to cross said isotransgenic lines produced in Example 4, for the purpose of producing commercial hybrids, according to techniques known to those skilled in the art and their knowledge

in terms of blooming for each parental elite line (Gallais A. et al., 1983).

Example 6: Determination of the genome of insertion of the transgene

13 events were obtained after transformation of hybrid embryos according to the protocol described above in Example 2. These events were then analyzed by Southern, to determine the number of copies of the transgene integrated, as described above in Example 3(a). 3 of these events proved to be monocopy.

The transformant 152-2E, chosen for the genomic border recovery described below, was obtained after transformation with the pRec 290 recombinant superbinary plasmid derived from pBIOS 290, derived from pBIOS 273 by integrating, at the XhoI site, a cassette "Pro HMWG-PhytI-Nos 3' - Pro HMWG-PhytII-Nos 3'". Said cassette is obtained according to conventional cloning techniques, using the wheat Pro HMWG sequence (Roberts et al The Plant Cell. 1 :569-578, 1989), the PhytI (accession No. EMBL, GenBank: AJ223470) and PhytII (accession No. EMBL, GenBank: AJ223471) nucleotide sequences and the 3' Nos sequence (Depicker et al., Mol. Gen. Genet. 235 (2-3): 389-396, 1992), and suitable restriction enzymes.

The recovery of the genomic borders was carried out on the right border (RB) side by the anchored PCR technique using the Genome Walker kit (Clontech laboratories inc.,Palo Alto, California). As

described in Example 3 (b), the identification of the genomic sequences adjacent to the inserted T-DNA comprises the following steps:

The GSPRB3/AP1 pair of oligonucleotides made it possible to carry out the first PCR on DNA of the transformant 152-1E digested with EcoRV. The product of this amplification was then subjected to a second PCR with the GSPRB9/AP2 pair of oligonucleotides.

The characteristics of the GSPRB3 and GSPRB9 oligonucleotides are described in the table of Example 3 and correspond to the attached sequence ID Nos. 18 and 24.

The AP1 and AP2 oligonucleotides are those provided in the Genome Walker kit and the PCR conditions are those recommended by the manufacturer Clontech. The 380 bp border fragment obtained in this final step was cloned into the pGEMT vector (Promega corporation, Madison, Wisconsin), in order to be amplified and used as a probe.

The identification of the recipient genome described in Example 3 (c), using the recovered border fragment, consists in hybridizing this probe fragment on an electrophoresis gel transfer containing the DNA of the transformant 152-2E digested with EcoRV and also the DNA of the 2 parental lines of the hybrid (A188 and L2) and that of 6 other transformants.

The result of the hybridization is represented on Figure 3: on this autoradiograph, DNA

corresponding to 7 different transformants is visualized, in the knowledge that there are from 1 to 3 plants per transformant.

The hybridization with the border probe reveals 1 band specific to the A188 genotype (1.7 Kb) and 1 specific for the elite line L2 (2.5 Kb). These 2 bands are found in all the transformants (proving that they are derived from the hybrid), except for the event t152-1E, which, itself, exhibits a lower band at approximately 0.8 Kb. It is deduced therefrom that the transgene is inserted into the expected 1.7 Kb EcoRV fragment of the genome of the A188 line.

This experiment therefore confirms the possibility of identifying, according to the protocol described, the genome of insertion of the T-DNA in the case of a transformant produced by the hybrid transformation technique, the A188 genome in this precise case.

It is also possible to obtain, according to the same protocol, transformants for which the insertion of the T-DNA takes place in the elite genome, with a probability of 1 transformant out of 2 on average.

When the genome of the elite parent is identified as being the genome which has received the insertion of the T-DNA, backcrosses may be carried out with the parent of interest and tested by selection

until isotransgenic lines are produced, as described above in Example 4.

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